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DEPARTMENT OF THE ARMY
Fort Detrick
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Chemical determination of the biologically active factor in the ribonucleic acid of tobacco mosaic virus.

by H. Schuster and G. Schramm.

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The tobacco mosaic virus(TMV) consists of a hollow protein cylinder 3,000 A in length, containing ribonucleic acid (RNA). The particle weighs $40 \cdot 10^6$ (1). Assuming an RNA content of 5.6%, the RNA portion's molecular weight amounts to $2.2 \cdot 10^6$. RNA may be separated from the protein by different methods. A particularly simple procedure consists in extracting the aqueous solution of TMV with phenol. The protein changes into the phenol phase thereby, while protein-free RNA remains quantitatively in the aqueous phase (2). RNA is infectious by itself (3). It represents the genetic material of the virus, effecting the production of specific viral protein in the host cell. Next to smaller decomposition products, the RNA obtained in this manner contains a homogeneous, high-molecular component. Measurements of sedimentation and viscosity give this main component a molecular weight of $2 \cdot 10^6$ (4). The total amount of nucleotides present in TMV, numbering about 6,000, is therefore combined in a single nucleotide chain. Differential ultracentrifugation showed that the high-molecular component only is infectious. Gierer (4) examined the question whether the entire RNA molecule with a mol. wt. of $2 \cdot 10^6$ is necessary for biological effectiveness, or whether other, smaller infectious factors existed. It was demonstrated that degradation products maintained by short treatment with RNase, elevated temperature or urea ceased to be infectious. The most distinct results were obtained upon cleavage with RNase. Measurements of the rate of inactivation in biological tests and simultaneous determination of the number of split phosphate bridges revealed that, on an average, the cleavage of any one of 6,000 nucleotides suffices to destroy the molecule's effectiveness. The intact RNA molecule therefore is required for the production of the specific virus protein in the infected cell. These tests do not exclude the possibility, however, that genetically inert ranges might be present in RNA.

The present investigation was to examine how the activity could be influenced by chemical reactions of purines and pyrimidines, while maintaining the cohesion of the whole string. In the event that nucleotides occur in RNA which are insignificant for genetic information, it ought to be possible to change a number of nucleotides without affecting infectiousness thereby.

Since high-molecular RNA is very labile, the possibilities of chemical transformation in which degradation is prevented, are limited; extreme pH values, elevated temperatures and extended reactions are to be avoided. It is necessary, in addition, to insure that the reaction leads to chemically well-defined and uniform reaction products which are easy to define quantitatively. Various preliminary tests indicated

the reaction of RNA with HNO_2 to be most suitable. As the structural diagram depicted in Fig. 1 shows, of the four different purine and pyrimidine residues, adenine, guanine and cytosine possess free amino groups which may be replaced by hydroxyl groups upon reaction with HNO_2. The transformation may be traced quantitatively by determining the amount of reacted purine and pyrimidine chromatically, after the hydrolysis of RNA. The effect of HNO_2 turns adenine into hypoxanthine, guanine into xanthine and cytosine into uracil. Derivatives are therefore formed from the purines, which normally do not occur in RNA, while the amount of already present uracil is increased by the transformation of cytosine. We established by measurements of sedimentation and by other tests that the molecule did not split in reacting with HNO_2. The conditions for the accomplishment of the task were thus achieved. If the conversion of an amino group to a hydroxyl group is designated as a hit, the question may be formulated as to how many hits are necessary for the inactivation of the infectious RNA molecule.

Methods.

1. Production of RNA.

RNA was produced according to the method of phenol extraction previously described (2). About 5% TMV in aqueous solution was shaken with an equal volume of 80% phenol for 20 minutes under cold conditions, the suspension was centrifuged, causing the division of phenol and the aqueous phase. The aqueous phase was twice more agitated with phenol, and the phenol was finally extracted from the aqueous phase with peroxide-free ether. The ether dissolved in the water was removed by N_2.

This procedure yields an aqueous solution of RNA of about 0.2-0.4%, the activity of which may be maintained for several weeks by storage at -10°C. Diluted solutions of RNA may be concentrated by precipitation of RNA with 2 volumes of ethanol and repeated dissolution of RNA in a smaller volume of water or buffer, without resulting in an essential loss of activity.

2. Reaction with HNO_2.

The reaction of RNA with HNO_2 was accomplished at pH values between 4.1 and 4.3 in an incubator at 21.5 ± 0.3°C. The test material, with a volume of 50 to 100 cc, contained the following components: 0.075% RNA, 0.25-m acetate buffer and 1-m $NaNO_2$. The quantitative data refer to the final concentrations per lot. The desired pH value was maintained during the reaction by addition of 25% acetic acid through an auto-titrator (model Radiometer Kopenhagen). Since free HNO_2 is labile and the NO forming upon its decomposition escapes, acetic acid must constantly be re-supplied. The concentration of nitrite during the test was determined spectro-photometrically by measuring the absorption at 230 millimicrons after strong dilution. A few drops of chloroform were added to prevent bacterial growth. In order to test the influence of hydrogen ion and salt concentrations on the infectiousness of RNA, a control test was made in which the RNA was maintained in a solution with

the same salt concentration and identical pH at the same temperature as that used in the reaction with HNO_2; however, $NaNO_2$ was replaced by a like amount of $NaCH_3CO_2$. In order to determine infectiousness and RNA bases, samples were taken from both lots at certain intervals.

3. Quantitative determination of the biological activity of RNA.

The determination of RNA activity was conducted essentially according to previous data (3) on Nicotiana glutinosa. The samples taken from the reaction solution were strongly diluted with 0.1-m phosphate buffer (pH 7); the reaction solution was thereby neutralized, so that further reaction with HNO_2 was immediately interrupted. The RNA concentration after dilution amounted to 60-150 γ/cc. Each solution was at once applied to 5 plants with 5 leaves each, with the aid of a glass spatula. After application, the remaining solution was rinsed from the leaves with tap water. The local lesions were counted after three to four days.

4. Determination of purines and pyrimidines.

We utilized the hydrolysis method of Smith and Markham (5) for the determination of purines and pyrimidines, in which the purines are obtained in free form and the pyrimidines as nucleotides. The samples of RNA taken from the materials were immediately neutralized, then the salt was removed by dialysis against water, which had been adjusted to pH 7 by addition of NH_3. The dialysed solution was evaporated, taken up by 1-n HCl (terminal concentration 10 mg RNA/cc 1-n HCl) and hydrolysed for 1 hour at 100°C. Upon acid hydrolysis of RNA treated with HNO_2, the following 6 cleavage products are obtained: adenine (Ad), Guanine (Gu), hypoxanthine (Hyx), xanthine (X), cytidylic acid (Cya) and uridylic acid (Ura).

Since a quantitative separation of the hydrolytic products in a single system of chromatography or by two-dimensional chromatography was impossible, four different systems had to be used, as shown in Table 1. Each product of hydrolysis was determined in that chromatographic system in which its R_f value strongly deviated from the R_f values of all other products of hydrolysis.

0.1-m KOH proved to be the chromatographic system best suited to the individual fixation of Ad and Gu (Fig. 2a). In the system described by Levenbook (6): H_2O / NH_3, pH 10, the R_f values of the hydrolytic products were not always reproducible and the zones were less defined than in 0.1-m KOH.

The individual determination of Hyx was accomplished with Carter's (7) system: Secondary sodium phosphate/isoamyl alcohol (Fig. 2 b). X was established in the system 1-m acetic acid (Fig. 2 c). In this connection the amount of X applied should not be too large, since indistinct zones develop owing to the poor solubility of X in acid solutions; besides, the amount applied will not be returned quantitatively.

Since the individual determination of X was connected with difficulties, Hyx and X were also analysed as a sum. The total analysis was more precise, since Hyx and X in the system 0.1-m KOH have the same Rf value (Fig. 2 a) and X is easily soluble in o.1-m KOH.

Since the naturally occurring bases of purine and pyrimidine do not decompose in the acid hydrolysis of RNA, we demonstrated in a control test that Hyx and X are not destroyed either under the conditions of hydrolysis.

We used Wyatt's (8) system of isopropanol-HCl in the individual determination of Cya and Ura. As revealed by Fig. 2 d, the two zones are well separated from the other products of hydrolysis.

The observed Rf values as well as the elution agents and extinction values used in analysis are summarized in Table 1. The extinction values listed are partially extracted from the literature, partly determined by means of standard substances. 0.05 or 0.1 cc of the hydrolytic mixture was used for each chromatography, corresponding to 500 to 1,000 γ products of hydrolysis. After the development of the chromatograms the papers were dried and the zones were localized with the aid of a low pressure mercury lamp NK 20/40 of the Quartz Lamp Company Hanau and the Schottfilter UG 5. The zones were cut out and treated with the elution agents indicated. The times of elution were 45 min. for 0.1-n HCl (temp. 45°) and 60 min. for 0.1-n KOH (20°C). At the level of the zones, strips of paper of equal size were cut out and their eluates were used as blank values in spectro-photometric analysis. In the use of 0.1-n KOH as elution agent, very high blank values were obtained, necessitating the washing of the paper used for chromatography. For this purpose the sheets were suspended in 0.1-n KOH for 60 min. at 30-40°C, then washed with dist. water and dried. Schleicher-Schnell paper 2043 b was used in all systems of chromatography. In order to compare analytical values, they were converted to 4 moles (2.2 moles purine and 1.8 mole pyrimidine, corresponding to the basic ratio of TMV-RNA).

Results.

1. Determination of the rate of reaction.

The reaction with amino groups depends on the concentration of undissociated HNO_2. Since the pH value of nitrous acid at 21°C is close to 3.4, it may be expected at pH values $\gg$ 3.4 (i.e. as long as the amount of nitrite ions may be considered as being practically constant) that the rate of reaction is proportional to the H ion concentration. It is necessary, therefore, to maintain a certain pH value during experiments. In the following, two examples of tests shall be discussed in detail. The graphic representation of the first test, conducted at pH 4.3, shows that the amount of Ad and Gu drops off linearly with time and that the sum of X and Hyx increases at the same rate (Fig. 3 a). Since the total of purines remained constant during the reaction (within the margins of error) and no other components were observed on the chromatogram, other than the expected products of hydrolysis, no measurable side reactions

occur, with the exception of desamination. The same is true of the transformation of Cya (Fig. 3 b). Here, too, the decrease in Cya corresponds to an increase in Ura. The obtained rates of reaction are summarized in Table 2. The low rate of Cya is due partly to the circumstance that the fraction of this base in RNA is smaller than that of each of the two purines. As the second column shows, the rates α/m, applied to the same molar relation, are of approximately identical size. The nearly linear progression of the curves is explained by the fact that the concentration of HNO_2 remains constant during the reaction in view of the large surplus of $NaNO_2$, and that the reacted quantity of RNA does not exceed 20%.

Owing to the poor solubility of X in 1-m CH_3COOH, the molar relation of Hyx/X always yielded values that where slightly too high, lying between 1.2 and 1.6. We therefore relied on the precise total determination of X + Hyx in our computations.

Fig. 4 a and b reflect an identical test at pH 4.1. The hydrogen ion concentration at 4.1 is larger than at pH 4.3 by the factor 1.6. The rate of reaction was elevated by nearly the same factor (see Table 2). Here, too, the gain in reaction products corresponds precisely to the loss in starting products.

2. Determination of molar weight.

In order to insure that the inactivation of RNA is caused solely by reaction of the NH_2 groups and not by cleavage of the nucleotide chain, it was tested whether a degradation had taken place during reaction with HNO_2. Control expsriments, in which RNA was subjected to the same pH and the same salt concentration in the absence of nitrite, initially showed that no inactivation occurs. Examinations in the ultra-centrifuge revealed correspondingly that the sedimentation constant is not changed thereby. RNA treated for 80 min. with nitrite was similarly examined in the ultracentrifuge. In order to remove the salt, RNA was settled out by addition of ethanol and reabsorbed in 0.02-m phosphate buffer with pH 7.0. Even after such a lengthy reaction, no change was observed in the sedimentation constant, while the activity had dropped to about 1/10 of the starting value in the first 30 min. In both cases $s_{20} = 19$ S was obtained for a 0.3% solution in 0.02-m phosphate with pH 7. In order to eliminate other non-specific cleavages of RNA with HNO_2, a preparation treated with HNO_2 for 72 hours at pH 4.3 was dialysed after the reaction and the dialysate was freed of HNO_2 and examined spectro-photometrically for RNA degradation products. Such products were not found, however.

3. Rate of inactivation.

The same lots which had served in the observation of the reaction of bases with nitrite, were used for the biological determination of decrease in infectiveness of RNA. The results of inactivation tests at pH 4.1 and 4.3 are given in Table 3. Under the influence of nitirite the number of lesions drops off steeply, while the control, at identical pH

and salt concentration without nitrite, retained its original activity within the margins of fluctuation of the test. It is noteworthy that the rate of inactivation also rises at a higher H ion concentration, in consonance with the rate of reaction of nitrous acid. The graphic representation (Fig. 3 c and 4 c) offer a better perspective on the course of reaction, in which the natural logarithm of the number of lesions is entered against the time. Since the logarithm of the number of lesions at moderately high RNA concentrations is proportional to the logarithm of the concentration of infectious RNA, the linear progression of these curves shows that the concentration of infectious RNA decreases exponentially with time. The inactivation of RNA therefore takes place at any given time by the change of a single nucleotide, and the procedure corresponds to the theory of the one-hit process. In Fig. 3 c the exponential drop in the number of lesions only apparently fails to proceed from the time t = 0, since due to the high number of lesions at the start, the proportionality to the concentration of infectious RNA is no longer assured.

4. Computation of the sensitive range of RNA.

If, on the average, the desamination of one of a total of N nucleotides in RNA leads to inactivation, then the decrease in infectiousness in the course of time follows the equation

$$I_t = I_0 e^{-N\alpha t} \qquad (1a)$$

$$\ln I_0 - \ln I_t = N\alpha t \qquad (1b)$$

with I_t = infectiousness at time t, I_0 = infectiousness at time t = 0, α = probability of the chemical change of an arbitrary nucleotide in the unit of time, t = reaction time.

In order to determine the number N, time t is first obtained from the drop in infecticusness, after which it has dropped to 1/e, i.e. when $\ln I_0 - \ln I_t = 1$. It amounts to

t_1 = 9 min. (pH = 4.3),
t_2 = 3.65 min. (pH = 4.1).

Within these times each active RNA molecule has therefore received an average of one hit.

In order to determine the probability of the change of one nucleotide, α, the reaction rates of the four nucleotides were added together, and the rate of Ura, which cannot be changed by HNO_2, was equated with zero. The sum was then divided by four. This resulted in:

$$\alpha_1 = \frac{[11.5\ (Ad + Gu) + 3.0\ (Cya)] \cdot 10^{-5}}{4} = 3.6 \cdot 10^{-5}.$$

$$\alpha_2 = \frac{[26.6\ (Ad + Gu) + 4.6\ (Cya)] \cdot 10^{-5}}{4} = 7.8 \cdot 10^{-5}.$$

If α_1 and t_1 or α_2 and t_2 are inserted in equation (1 b), $N_1 = 3{,}100$ and $N_2 = 3{,}500$ are obtained.

Accordingly, one hit on a mean of 3,300 nucleotides causes the loss of infectiousness of the entire molecule. Since TMV RNA with a mol. wt. of about $2 \cdot 10^6$ consists of a total of 6,000 nucleotides, this would mean that at least one half of all nucleotides are necessary for biological effectiveness, or, expressed differently, one hit per RNA molecule inactivates it with 50% probability. It follows, on the other hand, that a certain portion of the nucleotides may be desaminated without loss of biological activity. Nothing may be said regarding the question, which nucleotides are required for activity and which are not. Since the number of sensitive nucleotides is in excess of 3,000, but only 1,600 are present of each type of necleotide, it may be assumed that the hits on one of these types of nucleotides only have an inactivating effect. Thus, for instance, hits on Ad and Gu or on Ad and Cya or on Gu and Cya ought to inactivate.

The results could also be harmonized with the assumption that the transformation of Cya or a part thereof into naturally occurring Ura does not lead to inactivation, but to mutation. Even the desamination of Gu residues could result in replicatable derivatives, in the event only the substituent in the 6 position, which is not attacked, is essential for the biological effect. Tests are presently being conducted elsewhere to determine the extent to which mutants appear after treatment with HNO_2. In the event these investigations lead to a positive result, it would be possible that each nucleotide influences the biological effectiveness of RNA. Every change would then lead either to inactivation or to mutation. It may be deduced from results reported in this paper that undoubtedly more than one half of all nucleotides contained in RNA are necessary for the transfer of genetic information. It follows, in agreement with the degradation tests conducted by Gierer (4), that the existence of a smaller fraction of RNA, infectious by itself, is improbable. If this should occur at all, it would have to include at least half of all nucleotides occurring in the whole RNA molecule.